Proc. Natl. Acad. Sci. USA
Vol. 73, No. 10, pp. 3719–3722, October 1976
Pathology

Vitamin A-containing lipocytes and formation of type III collagen in liver injury

(fibrosis/reticulum/Ito cells/immunofluorescence microscopy)

GEOFFREY KENT*, STEFFEN GAY†, TOHRU INOUYE*, RAJA BAHU*, O. T. MINICK*, AND HANS POPPER‡

* Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611; † Max-Planck-Institut für Biochemie, Abt. Kühn, D-8033 Martinsried/München, West Germany; and ‡ Stratton Laboratory for the Study of Liver Diseases, Mount Sinai School of Medicine of the City University of New York, New York, N.Y. 10029

Contributed by Hans Popper, July 22, 1976

ABSTRACT **Hepatocellular necrosis in carbon tetrachloride-induced injury of rats is associated with an accumulation of lipocytes (perisinusoidal cells or Ito cells) containing fat droplets and giving vitamin A fluorescence. In the subsequent formation of connective tissue septa, transitional cells having morphologic characteristics of lipocytes and fibroblasts are abundant and are associated with the appearance of type III collagen. The features suggest that the lipocyte is the precursor of the fibroblasts responsible for parenchymal fibrillogenesis and under these conditions forms type III collagen. The process is a postulated link between hepatocellular necrosis and fibrosis.**

Hepatic fibrosis is a key factor in the chronicity of liver disease and the development of cirrhosis. Although the excessive accumulation of fibers in the hepatic parenchyma may be caused by collapse of the preexisting framework, new formation of collagen as a rule complicates such collapse and is often the primary event (1). While fibrogenesis in the portal fields shares morphologic characteristics with similar processes elsewhere in the body, intralobular fibrogenesis is less well understood and the cells engaged in this process are at present not defined. In recent years, attention has been focused, mainly as a result of electron microscopic studies, upon the perisinusoidal cells [Ito cells (2, 3) or lipocytes (4)] located in the Space of Disse between the sinusoidal endothelium and hepatocytes. These cells contain small fat droplets displaying strong vitamin A fluorescence and some rough endoplasmic reticulum but, in contrast to Kupffer cells, are free of phagosomes. Morphologic investigations have suggested that they play a role in vitamin A metabolism (5, 6) and are resting precursors of parenchymal fibroblasts (7–11). To substantiate the last thesis, we investigated the behavior of these cells in acute and chronic stages of carbon tetrachloride (CCl_4) intoxication. Since preliminary studies had indicated that loading doses of retinol increase the number of fat droplets in the lipocytes and thus make them more conspicuous on microscopic examination, supplements of retinol were used as markers for perisinusoidal cells in rats similarly treated. Since different types of collagen varying in amino acid sequence (12) have recently been characterized, and the presence of at least two types in the liver has been demonstrated by biochemical and immunofluorescent techniques (13, 14), the types of collagen related to lipocytes were investigated in the present study.

MATERIALS AND METHODS

Groups of eight Sprague-Dawley rats on Purina diet received subcutaneous injections of 1 ml of CCl_4 in olive oil per kilogram of body weight twice weekly for 1, 2, 6, 12, and 24 weeks; another group received in addition 12,500 international units of retinyl acetate per kilogram of body weight given subcutaneously 5 days a week for the duration of the experiments. A third group received only 12,500 international units of retinyl acetate per kilogram; a fourth control group received neither CCl_4 nor vitamin A. The animals were sacrificed at the end of each experimental period. The livers were examined by (*a*) routine histologic techniques; (*b*) fluorescence microscopy of nonfixed cryostat sections for rapidly fading green autofluorescence characteristic of vitamin A, and for fat stained by Phosphin-3R (5); (*c*) light microscopy of 1 μm thick Epon-embedded sections permitting identification of lipocytes; (*d*) electron microscopy; and (*e*) immunofluorescence microscopy with collagen type-specific antibodies tagged with fluorescein. The antibody against type I collagen is primarily directed toward a site in the carboxy-terminal portion of type I collagen; the antibody against type III collagen is directed toward several sites in the helical and nonhelical portions of the molecule; and the antibody against type III procollagen is directed toward the amino-terminal region (15, 16).

RESULTS

Vitamin A supplementation resulted in prominence of lipocytes without significantly altering the light microscopic appearance of paraffin-embedded material. The total amount of collagen estimated by determination of hepatic hydroxyproline was the same in rats supplemented with vitamin A and rats not supplemented, the collagen concentration increasing in CCl_4-treated animals from 1.00 mg to 1.71 mg of hydroxyproline per g of dried, defatted tissue at the end of 6 weeks, and from 1.17 mg to 5.03 mg of hydroxyproline after 12 weeks.

Light and electron microscopy

After 1 or 2 weeks' duration of the experiments, the livers displayed centrolobular necrosis with steatosis in the surrounding parenchyma. The necrotic area contained, besides macrophages, conspicuous numbers of lipocytes, as shown by many Phosphin-3R-positive fat droplets imparting a fading vitamin A autofluorescence. Lipocytes were also recognized in 1 μm thick sections and under the electron microscope. They were far more conspicuous, and indeed presented a striking appearance, after vitamin A supplementation (Fig. 1), when the cells appeared larger and contained a greater number of fat droplets, the latter imparting a strong vitamin A autofluorescence. They were intermixed with pigmented macrophages containing granules giving a yellow, nonfading autofluorescence, presumably of lipofuscin. Fibroblasts were not conspicuous in the necrotic area in the first 2 weeks.

In animals treated for longer periods, connective tissue septa linking central zones with each other were observed after 6 weeks; after 12 and 24 weeks, the septa extended to the portal

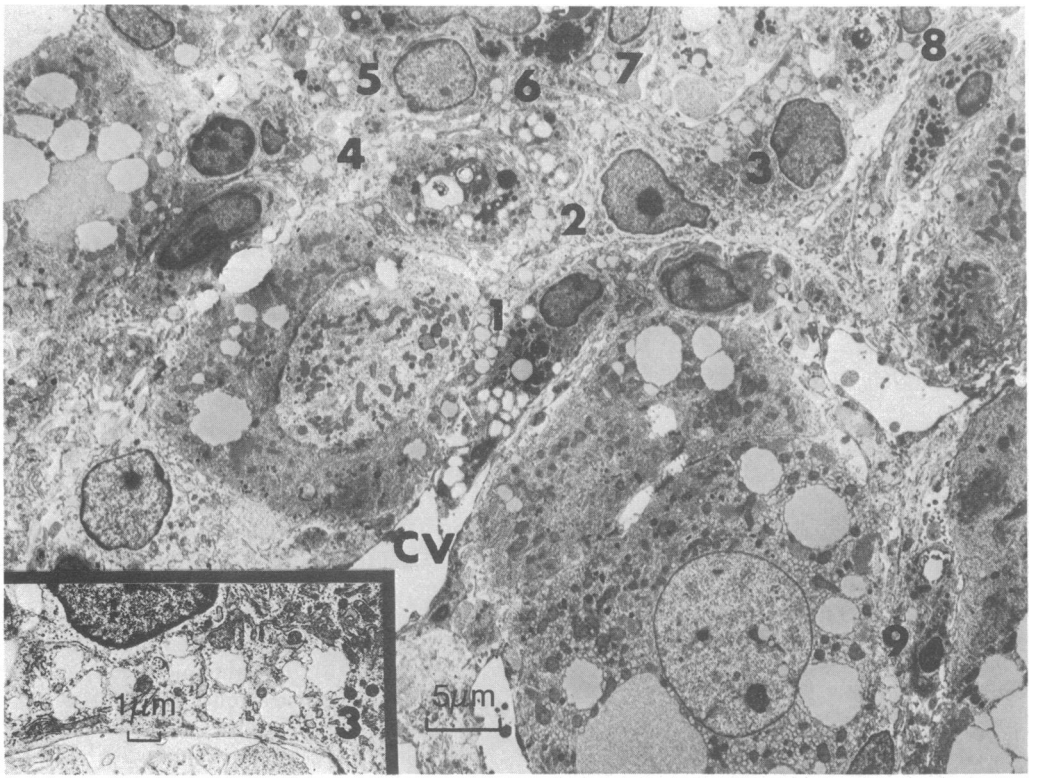

FIG. 1. Electron micrograph of liver from rat receiving vitamin A and CCl_4 for 2 weeks. Note prominent accumulation of lipocytes (nos. 1 to 9) in area of centrolobular necrosis. CV, central vein. Magnification ×1800. (*Inset*) Cell (no. 3) at higher magnification showing lipid droplets. Magnification ×4400.

tracts, and a few hepatocytic regenerative nodules were observed. The septa displayed a few bile ductules, macrophages containing autofluorescent lipofuscin granules, and lipocytes, which were particularly conspicuous in animals receiving vitamin A supplements (Fig. 2). The electron microscope showed typical fibroblasts as well as lipocytes characterized by abundant rough endoplasmic reticulum, the dilated cisternae of which contained a fluffy material. In addition, the septa contained many cells that had vitamin A fluorescent fat droplets characteristic of lipocytes in part of their cytoplasm and conspicuous rough endoplasmic reticulum as seen in fibroblasts in other parts, with gradations in these features. These cells, apparently transitional forms between lipocytes and fibroblasts, were surrounded by layers of collagen. Both lipocytes and transitional cells were more conspicuous in animals supplemented with vitamin A. In the animals treated with CCl_4 for 24 weeks, the number of fibroblasts exceeded that of the lipocytes, and transitional cells were more frequent.

In animals in which vitamin A supplements had been discontinued 2 weeks before the examination, the number of fat droplets was smaller and vitamin A fluorescence less conspicuous. Occasional mitoses were seen in the lipocytes in the early stages of the experiment; they were not encountered in the later stages.

Immunofluorescent studies

Immunofluorescent studies of control livers with antibodies against collagen revealed type I collagen in portal tracts and only small bundles, irregularly distributed, in the parenchyma (Fig. 3A). Type III collagen was observed as a loose network throughout the lobule and was also seen in portal fields and around central veins (Fig. 3B). This network, which stains with antibody against type III procollagen as well as antibody against type III collagen, appeared identical with the reticulum fibers demonstrated by silver impregnation. After 2 weeks of treatment with CCl_4, the accumulation of lipocytes in the centrolobular zone was accompanied by type III collagen, and the distribution of type III collagen was again identical with that of the fibers demonstrated by silver impregnation. The distribution of type I collagen, by contrast, remained unchanged. The septa at 6 weeks' duration of the experiment consisted mainly of reticulum fibers and reacted with type III collagen antibody (Fig. 4A); some type I collagen bundles were also seen in septa (Fig. 4B).

DISCUSSION

Centrolobular CCl_4 necrosis is associated with an accumulation of cells having vitamin A autofluorescence and showing the morphologic characteristics of lipocytes. Such apparent chemotactic effect was perhaps stimulated by breakdown products of liver cells directly or by macrophages accumulating in the area of necrosis. The accumulation of lipocytes occurs at a time when biochemical parameters, such as raised proline hydroxlase (EC 1.14.11.2) activity (17) and increased synthesis of type III collagen in the liver, indicated active fibroplasia. A subsequent increase of fibroblasts is accompanied by the presence of cells with features transitional between lipocytes and fibroblasts, suggesting transformation of the former into the latter. Vitamin A supplementation does not significantly alter the process, but provides for greater prominence of lipocytes and transitional cells. The apparently greater number of both types of cells after vitamin A supplementation can probably be accounted for by their better morphologic visibility and by the stereologic principle that larger cells are seen more readily in section. The animals that had been loaded with vitamin A therefore permit better analysis of the on-going process. Although some obser-

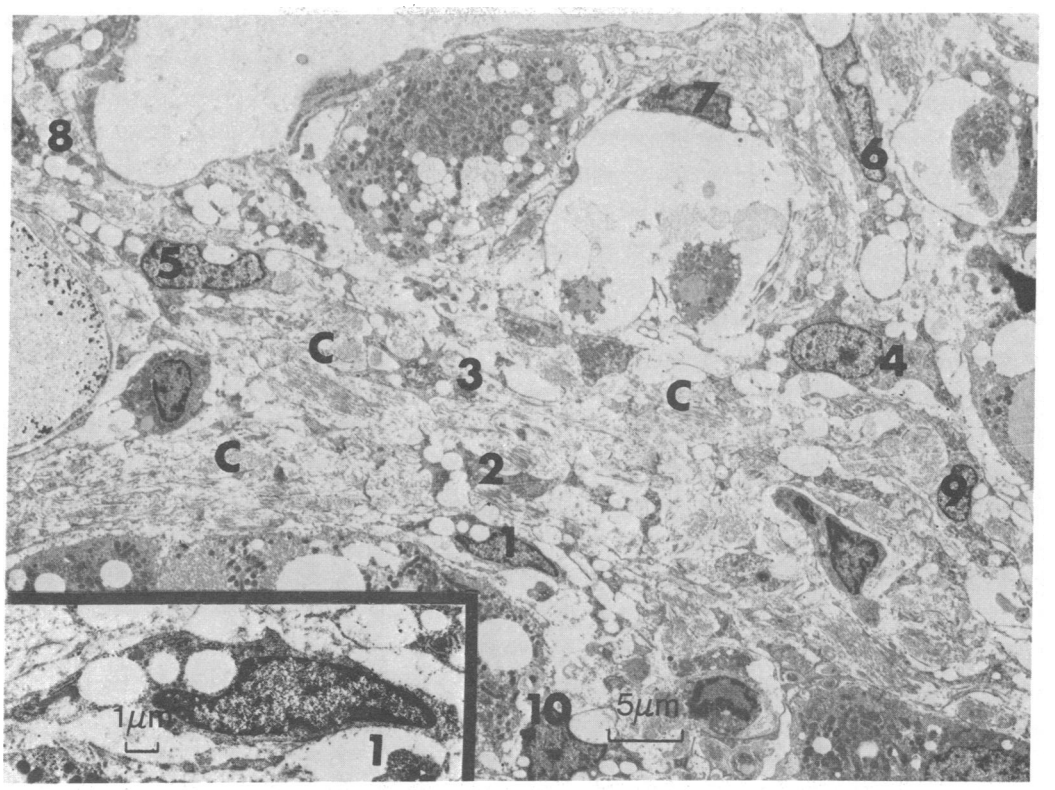

FIG. 2. Electron micrograph of septum in liver of rat receiving vitamin A and CCl_4 for 6 weeks. There are some fibroblasts and many lipocytes (nos. 1 to 10), some with morphologic features of fibroblasts in part of the cytoplasm. They are surrounded by collagen (C). The septa display strong, fading vitamin A autofluorescence. Magnification ×1700. (*Inset*) Cell (no. 1) at higher magnification showing lipid droplets as well as rough endoplasmic reticulum. Magnification ×4600.

vations suggest that hypervitaminosis A favors hepatic fibrosis in man (18), no evidence was obtained that the doses given to the rats increased fibroplasia. Vitamin A autofluorescence and fat droplets in lipocytes decreased as fibrosis proceeded, perhaps reflecting the role of vitamin A in the synthesis of glucosaminoglycans (19, 20).

The newly formed collagen in its initial stages appears to be essentially type III collagen and type III procollagen and parallels the presence of reticulum fibers. It is possible that type III procollagen influences the size of fibers formed by type III collagen (15). The abundance of type III collagen in later stages supports the impression that the lipocyte-fibroblast cell line is responsible for the formation of type III collagen at the stages studied. The increased content of type I collagen in the late stages of fully developed cirrhosis may indicate that the same cells can synthesize both types of collagen (16).

This work was supported in part by the National Institute for Occupational Safety and Health Contract no. 21-75-0044 and the Deutsche Forschung Gesellschaft Ga 218.1. We thank Drs. H. Nowack and R. Timpl for the preparation of antibodies.

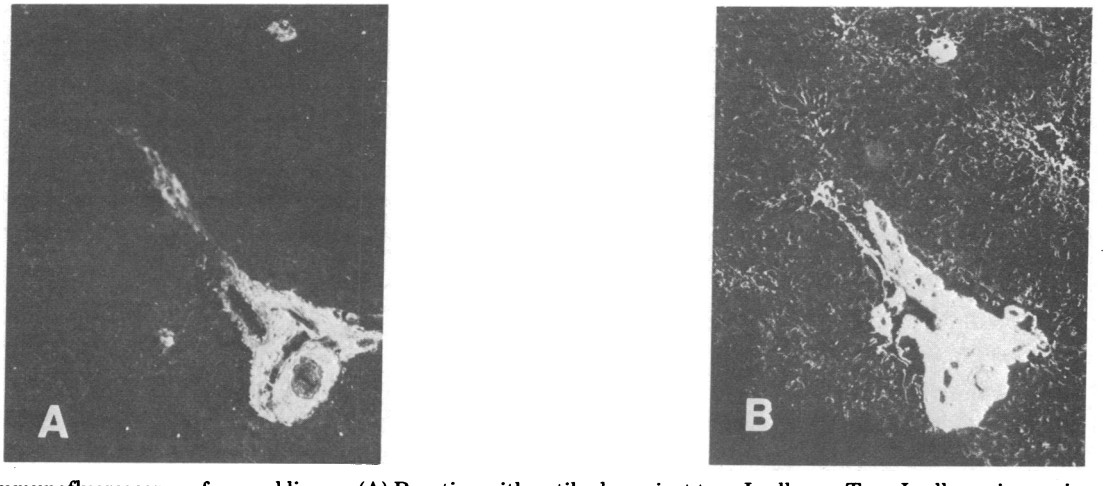

FIG. 3. Immunofluorescence of normal liver. (A) Reaction with antibody against type I collagen. Type I collagen is seen in a portal tract. The circular fluorescence in the upper right portion of the photograph marks a blood vessel. Magnification ×50. (B) Reaction with antibody against type III collagen. Note presence of type III collagen again in the portal tract, but also within the lobule as intralobular reticulum fibers. Magnification ×50.

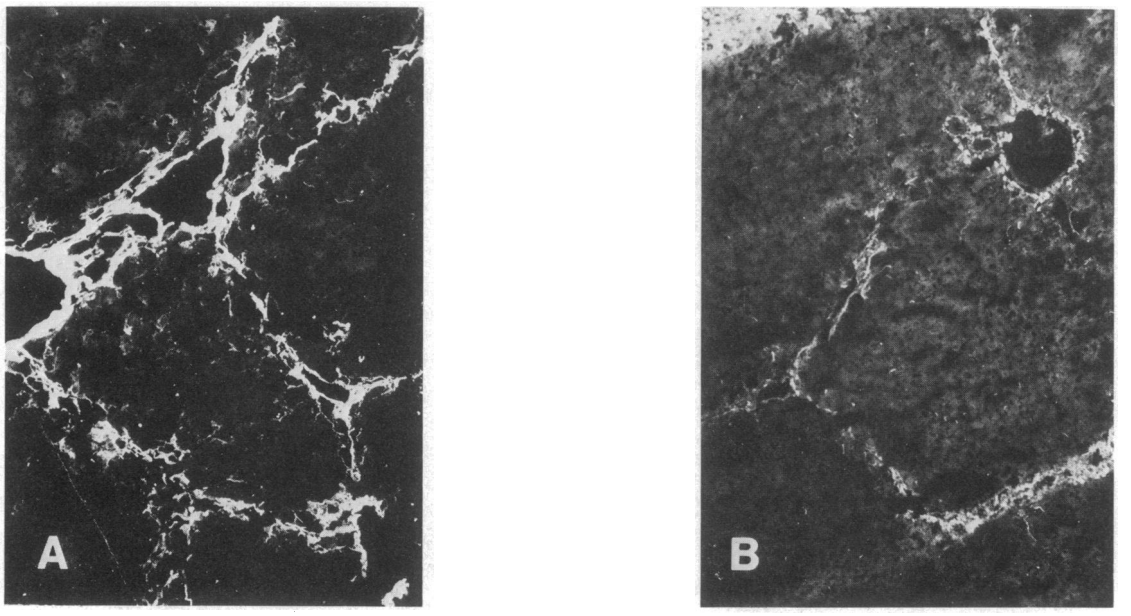

FIG. 4. Septum in liver of rats treated with CCl_4 for 6 weeks. (A) Strong immunofluorescence with antibody against type III collagen. Magnification ×50. (B) Immunofluorescence with antibody against type I collagen. Some type I collagen is present in the septa linking central with central as well as portal canals. Magnification ×50.

1. Popper, H. & Udenfriend, S. (1970) *Am. J. Med.* **49,** 707–721.
2. Ito, T. & Nemoto, M. (1952) *Okajima Folia Anat. Jap.* **24,** 243–258.
3. Ito, T. (1973) *Gunma Rep. Med. Sci.* **6,** 119–163.
4. Bronfenmajor, S., Schaffner, F. & Popper, H. (1966) *Arch. Pathol.* **82,** 447–453.
5. Popper, H. (1944) *Physiol. Rev.* **24,** 205–224.
6. Wake, K. (1974) *J. Cell Biol.* **63,** 683–691.
7. Schnack, H., Stockinger, L. & Wewalka, F. (1967) *Rev. Int. Hepatol.* **17,** 855–860.
8. McGee, J. O'D. & Patrick, R. S. (1972) *Lab. Invest.* **26,** 429–440.
9. Tanikawa, K. (1975) in *Collagen Metabolism in the Liver,* eds. Popper, H. & Becker, K. (Stratton Intercontinental Med. Bk. Corp., New York), pp. 93–99.
10. Popper, H. & Kent, G. (1975) *Clinics in Gastroenterology* **4,** 315–332.
11. Kent, G., Bahu, R., Inouye, T., Minick, T. & Popper, H. (1975) *Gastroenterology* **69,** 835.
12. Miller, E. J. & Matukas, V. J. (1974) *Fed. Proc.* **33,** 1187–1204.
13. Gay, S., Fietzek, P. P., Remberger, K., Eder, M. & Kuhn K. (1975) *Klin. Wochenschr.* **53,** 205–208.
14. Rojkind, M. & Martinez-Palomo, A. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 539–543.
15. Nowack, H., Gay, S., Wick, G., Becker, U. & Timpl, R. (1976) *J. Immunol. Meth.,* in press.
16. Gay, S., Martin. G. R., Muller, P. K., Timpl, R. & Kuhn, K. (1976) *Proc. Natl. Acad. Sci. USA,* **73,** in press.
17. McGee, J. O'D., O'Hare, R. P. & Patrick, R. S. (1973) *Nature New Biol.* **243,** 121–123.
18. Hruban, Z., Russell, R. M., Boyer, J. L., Glagov, S. & Bagheri, S. A. (1974) *Am. J. Pathol.* **76,** 451–468.
19. Rave, Von O., Wagner, H., Junge-Hulsing, S. & Hauss, W. H. (1972) *Z. Rheumaforsch.* **31,** 245–253.
20. Sudhakaran, P. R. & Kurup, P. A. (1974) *J. Nutr.* **104,** 871–883.